FATTY ACID EPOXYGENASE GENES FROM PLANTS AND USES THEREFOR IN MODIFYING FATTY ACID METABOLISM

RELATED APPLICATION DATA

5 This application is a continuation-in-part application of USSN 09/059,769 filed on April 14, 1998, which claims benefit of priority under Title 35, U.S.C. §119 from Australian Patent Application No. PO6223 filed on April 15, 1997 and Australian Patent Application No. PO6226 filed on April 15, 1997, and which also claims benefit of priority under Title 35, U.S.C. §119(e) from USSN 60/043,706 filed on April 16, 1997 and from USSN 60/050,403
10 filed on June 20, 1997.

FIELD OF THE INVENTION

The present invention relates generally to novel genetic sequences that encode fatty acid epoxygenase enzymes. In particular, the present invention relates to genetic sequences
15 that encode fatty acid Δ12-epoxygenase enzymes as defined herein. More particularly, the present invention provides cDNA and genomic gene sequences that encode plant fatty acid epoxygenases, in particular from *Crepis palaestina* or *Vernonia galamensis*. The genetic sequences of the present invention provide the means by which fatty acid metabolism may be altered or manipulated in organisms such as yeasts, moulds, bacteria, insects, birds,
20 mammals and plants, in particular to convert unsaturated fatty acids to epoxy fatty acids therein. The invention extends to genetically modified oil-accumulating organisms transformed with the subject genetic sequences and to the oils derived therefrom. The oils thus produced provide the means for the cost-effective raw materials for use in the efficient production of coatings, resins, glues, plastics, surfactants and lubricants, amongst others.

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GENERAL

Those skilled in the art will be aware that the present invention is subject to variations and modifications other than those specifically described herein. It is to be understood that the invention includes all such variations and modifications. The invention also includes all
30 such steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

Throughout this specification, unless the context requires otherwise, the word "comprise",
35 or variations such as "comprises" or "comprising", will be understood to imply the inclusion

of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

This specification contains nucleotide sequence information prepared using the program PatentIn Version 3.1 presented herein after the claims. Each nucleotide sequence is identified in the sequence listing by the numeric indicator <210> followed by the sequence identifier [e.g. <210>1, <210>2, etc]. The length, type of sequence [DNA, protein (PRT), etc] and source organism for each nucleotide sequence are indicated by information provided in the numeric indicator fields <211>, <212> and <213>, respectively. Nucleotide sequences referred to in the specification are defined by the term "SEQ ID NO: ", followed by the sequence identifier [e.g. SEQ ID NO: 1 refers to the sequence in the sequence listing designated as <400>1].

BACKGROUND TO THE INVENTION

There is considerable interest world-wide in producing chemical feedstock, such as fatty acids, for industrial use from renewable plant sources rather than from non-renewable petrochemicals. This concept has broad appeal to manufacturers and consumers on the basis of resource conservation and provides a significant opportunity to develop new industrial crops for agriculture.

There is a diverse array of unusual fatty acids in nature and these have been well characterized (Badam & Patil, 1981; Smith, 1970). Many of these unusual fatty acids have industrial potential and this has led to interest in domesticating such species to enable agricultural production of particular fatty acids.

One class of fatty acids of particular interest are the epoxy-fatty acids, consisting of an acyl chain in which two adjacent carbon bonds are linked by an epoxy bridge. Due to their high reactivity, they have considerable application in the production of coatings, resins, glues, plastics, surfactants and lubricants. These fatty acids are currently produced by chemical epoxidation of vegetable oils, mainly soybean oil and linseed oil, however this process produces mixtures of multiple and isomeric forms and involves significant processing costs.

Attempts are being made by others to develop some wild plants that contain epoxy fatty acids (e.g. *Euphorbia lagascae*, or *Vernonia galamensis*) into commercial sources of these oils. However, problems with agronomic suitability and low yield potential severely limit the commercial utility of traditional plant breeding and cultivation approaches.

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The rapidly increasing sophistication of recombinant DNA technology is greatly facilitating the efficiency of commercially-important industrial processes, by the expression of genes isolated from a first organism or species in a second organism or species to confer novel phenotypes thereon. More particularly, conventional industrial processes can be made
10 more efficient or cost-effective, resulting in greater yields per unit cost by the application of recombinant DNA techniques.

Moreover, the appropriate choice of host organism for the expression of a genetic sequence of interest provides for the production of compounds that are not normally
15 produced or synthesized by the host, at a high yield and purity.

However, despite the general effectiveness of recombinant DNA technology, the isolation of genetic sequences which encode important enzymes in fatty acid metabolism, in particular the genes which encode the fatty acid Δ12-epoxygenase enzymes responsible for
20 producing 12,13-epoxy-9-octadecenoic acid (vernolic acid) and 12,13-epoxy-9,15-octadecadienoic acid, amongst others, remains a major obstacle to the development of genetically-engineered organisms which produce these fatty acids.

Until the present invention, there were only limited biochemical data indicating the nature of
25 fatty acid epoxygenase enzymes, in particular Δ12-epoxygenases. However, in *Euphorbia lagascae*, the formation of 12,13-epoxy-9-octadecenoic acid (vernolic acid) from linoleic acid appears to be catalyzed by a cytochrome-P450-dependent Δ12 epoxygenase enzyme (Bafor *et al.*, 1993; Blee *et al.*, 1994). Additionally, developing seed of linseed plants have the capability to convert added vernolic acid to 12,13-epoxy-9,15-octadecadienoic acid by
30 an endogenous Δ15 desaturase (Engeseth and Stymne, 1996). Epoxy-fatty acids can also be produced by a peroxide-dependent peroxygenase in plant tissues (Blee and Schuber, 1990).

In work leading up to the present invention, the inventors sought to isolate genetic
35 sequences which encode genes which are important for the production of epoxy-fatty acids,

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such as 12,13-epoxy-9-octadecenoic acid (vernolic acid) or 12,13-epoxy-9,15-octadecadienoic acid and to transfer these genetic sequences into highly productive commercial oilseed plants and/or other oil accumulating organisms.

5 SUMMARY OF THE INVENTION

One aspect of the invention provides an isolated nucleic acid which encodes or is complementary to an isolated nucleic acid which encodes a fatty acid epoxygenase.

A second aspect of the invention provides an isolated nucleic acid which hybridizes under 10 at least low stringency conditions to at least 20 contiguous nucleotides of SEQ ID NOs:1 or 3 or 5 or 19 or 19, or a complementary sequence thereto.

A further aspect of the invention provides isolated nucleic acid comprising a sequence of nucleotides selected from the group consisting of:

15 (i) a nucleotide sequence that is at least 65% identical to a sequence selected from the group consisting of: SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, and SEQ ID NO: 19;

(ii) a nucleotide sequence that encodes an amino acid sequence that is at least about 50% identical to a sequence selected from the group consisting of: SEQ ID NO: 2, 20 SEQ ID NO: 4, SEQ ID NO: 6, and SEQ ID NO: 20; and

(iii) a nucleotide sequence that is complementary to (i) or (ii).

A further aspect of the invention provides a gene construct that comprises the isolated nucleic acid *supra,* in either the sense or antisense orientation, in operable connection with 25 a promoter sequence.

A further aspect of the invention provides a method of altering the level of epoxy fatty acids in a cell, tissue, organ or organism, said method comprising expressing a sense, antisense, ribozyme or co-suppression molecule comprising the isolated nucleic acid *supra* in said cell, 30 tissue, organ or organism for a time and under conditions sufficient for the level of epoxy fatty acids therein to be increased or reduced.

A further aspect of the invention provides a method of producing a recombinant enzymatically active epoxygenase polypeptide in a cell, said method comprising expressing

the isolated nucleic acid *supra* in said cell for a time and under conditions sufficient for the epoxygenase encoded therefor to be produced.

A further aspect of the invention provides a method of producing a recombinant enzymatically active epoxygenase polypeptide in a cell, said method comprising the steps of:

(i) producing a gene construct which comprises the isolated nucleic acid *supra* placed operably under the control of a promoter capable of conferring expression on said genetic sequence in said cell, and optionally an expression enhancer element;

(ii) transforming said gene construct into said cell; and

(iii) selecting transformants which express a functional epoxygenase encoded by the genetic sequence at a high level.

A still further aspect of the invention provides a method of producing a recombinant and enzymatically active epoxygenase polypeptide in a transgenic plant comprising the steps of:

(i) producing a gene construct which comprises the isolated nucleic acid *supra* placed operably under the control of a seed-specific promoter and optionally an expression enhancer element, wherein said genetic sequences is also placed upstream of a transcription terminator sequence;

(ii) transforming said gene construct into a cell or tissue of said plant; and

(iii) selecting transformants which express a functional epoxygenase encoded by the genetic sequence at a high level in seeds.

A further aspect of the invention provides a recombinant epoxygenase polypeptide or functional enzyme molecule.

A further aspect of the invention provides a recombinant epoxygenase which comprises a sequence of amino acids set forth in any one of SEQ ID NOs: 2 or 4 or 6 or 20 or 20 or a homologue, analogue or derivative thereof which is at least about 50% identical thereto. More preferably, the percentage identity to any one of SEQ ID NOs: 2 or 4 or 6 or 20 or 20 is at least about 65%.

A still further aspect of the invention provides a method of producing an epoxy fatty acid in a cell, tissue, organ or organism, said method comprising incubating a cell, tissue, organ or

organism which expresses an enzymatically active recombinant epoxygenase with a fatty acid substrate and preferably, an unsaturated fatty acid substrate, for a time and under conditions sufficient for at least one carbon bond, preferably a carbon double bond, of said substrate to be converted to an epoxy group.

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A further aspect of the invention provides an immunologically interactive molecule which binds to the recombinant epoxygenase polypeptide described herein or a homologue, analogue or derivative thereof.

10 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a linear representation of an expression plasmid comprising an epoxygenase structural gene, placed operably under the control of the truncated napin promoter (FP1; right-hand hatched box) and placed upstream of the NOS terminator sequence (right-hand stippled box). The epoxygenase genetic sequence is indicated by the right-hand open
15 rectangular box. The construct also comprises the NOS promoter (left-hand hatched box) driving expression of the *NPTII* gene (left-hand open box) and placed upstream of the NOS terminator (left-hand stippled box). The left and right border sequences of the *Agrobacterium tumefaciens* Ti plasmid are also indicated.

20 Figure 2 is a schematic representation showing the alignment of the amino acid sequences of the epoxygenase polypeptide of *Crepis palaestina* (Cpa12; SEQ ID NO: 2), a further epoxygenase derived from *Crepis sp.* other than *C. palaestina* which produces high levels of vernolic acid (*CrepX*; SEQ ID NO: 4), a partial amino acid sequence of an epoxygenase polypeptide derived from *Vernonia galamensis* (Vgal1; SEQ ID NO: 6), a full-length amino
25 acid sequence of an epoxygenase polypeptide derived from *Vernonia galamensis* (SEQ ID NO: 20), the amino acid sequence of the Δ12 acetylenase of *Crepis alpina* (Crep1; SEQ ID NO: 8), the Δ12 desaturase of *A. thaliana* (L26296; SEQ ID NO: 9), *Brassica juncea* (X91139; SEQ ID NO: 10), *Glycine max* (L43921; SEQ ID NO: 11), *Solanum commersonii* (X92847; SEQ ID NO: 12) and *Glycine max* (L43920; SEQ ID NO: 13), and the Δ12
30 hydroxylase of *Ricinus communis* (U22378; SEQ ID NO: 14). Underlined are three histidine-rich motifs that are conserved in non-heme containing mixed-function monooxygenases.

Figure 3 is a copy of a photographic representation of a northern blot hybridization showing
35 seed-specific expression of the *Crepis palaestina* epoxygenase gene exemplified by SEQ

ID NO: 1. Northern blot analysis of total RNA from leaves (lane 1) and developing seeds (lane 2) of *Crepis palaestina*. 15μg of total RNA was run on a Northern gel and blotted onto Hybond N^+ membrane from Amersham according to the manufacturer's instructions. The blot was hybridized at 60°C with a probe made from the 3' untranslated region of SEQ ID
5 NO: 1. The blot was washed twice in 2 x SSC (NaCl- Sodium Citrate buffer) at room temperature for 10 minutes, then in 0.1xSSC at 60°C for 20 min.

Figure 4 is a schematic representation of a binary plasmid vector containing an expression cassette comprising the truncated napin seed-specific promoter (Napin) and nopaline
10 synthase terminator (NT), with a *Bam*HI cloning site there between, in addition to the kanamycin-resistance gene *NPT*II operably connected to the nopaline synthase promoter (NP) and nopaline synthase terminator (NT) sequences. The expression cassette is flanked by T-DNA left border (LB) and right-border (RB) sequences.

15 Figure 5 is a schematic representation of a binary plasmid vector containing an expression cassette which comprises SEQ ID NO: 1 placed operably under the control of a truncated napin seed-specific promoter (Napin) and upstream of the nopaline synthase terminator (NT), in addition to the kanamycin-resistance gene *NPT*II operably connected to the nopaline synthase promoter (NP) and nopaline synthase terminator (NT) sequences. The
20 expression cassette is flanked by T-DNA left border (LB) and right-border (RB) sequences. To produce this construct, SEQ ID NO: 1 is inserted into the *Bam*HI site of the binary vector set forth in Figure 4.

Figure 6 is a graphical representation of gas-chromatography traces of fatty acid methyl
25 esters prepared from oil seeds of untransformed *Arabidopsis thaliana* plants [panel (a)], or *A. thaliana* plants (transgenic line Cpal-17) which have been transformed with SEQ ID NO: 1 using the gene construct set forth in Figure 5 [panels (b) and (c)]. In panels (a) and (b), fatty acid methyl esters were separated using packed column separation. In panel (c), the fatty acid methyl esters were separated using capillary column separation. The elution
30 positions of vernolic acid are indicated.

Figure 7 is a graphical representation showing the joint distribution of epoxy fatty acids in selfed seed on T_1 plants of Cpal2-transformed *Arabidopsis thaliana* plants as determined using gas chromatography. Levels of both vernolic acid (x-axis) and 12,13-epoxy-9,15-

octadecadienoic acid (y-axis) were determined and plotted relative to each other. Data show a positive correlation between the levels of these fatty acids in transgenic plants.

Figure 8 is a graphical representation showing the incorporation of ^{14}C-label into the chloroform phase obtained from lipid extraction of linseed cotyledons during labeled-substrate feeding. Symbols used; ♦, [^{14}C] oleic acid feeding; ■, [^{14}C] vernolic acid feeding.

Figure 9 is a graphical representation showing the incorporation of ^{14}C-label into the phosphatidyl choline of linseed cotyledons during labeled-substrate feeding. Symbols used; ♦, [^{14}C] oleic acid feeding; ■, [^{14}C] vernolic acid feeding.

Figure 10 is a graphical representation showing the incorporation of ^{14}C-label into the triacylglycerols of linseed cotyledons during labeled-substrate feeding. Symbols used♦, [^{14}C] oleic acid feeding; ■, [^{14}C] vernolic acid feeding.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

One aspect of the present invention provides an isolated nucleic acid which encodes or is complementary to an isolated nucleic acid which encodes a fatty acid epoxygenase.

Wherein the isolated nucleic acid of the invention encodes an enzyme which is involved in the direct epoxidation of arachidonic acid, it is particularly preferred that the subject nucleic acid is derived from a non-mammalian source.

As used herein, the term "derived from" shall be taken to indicate that a particular integer or group of integers has originated from the species specified, but has not necessarily been obtained directly from the specified source.

The term "non-mammalian source" refers to any organism other than a mammal or a tissue or cell derived from same. In the present context, the term "derived from a non-mammalian source" shall be taken to indicate that a particular integer or group of integers has been derived from bacteria, yeasts, birds, amphibians, reptiles, insects, plants, fungi, moulds and algae or other non-mammal.

In a preferred embodiment of the present invention, the source organism is any such organism possessing the genetic capacity to synthesize epoxy fatty acids. More preferably,

the source organism is a plant such as, but not limited to *Chrysanthemum spp., Crepis spp., Euphorbia spp.* and *Vernonia spp.,* amongst others.

Even more preferably, the source organism is selected from the group consisting of: *Crepis*
5 *biennis, Crepis aurea, Crepis conyzaefolia, Crepis intermedia, Crepis occidentalis, Crepis palaestina, Crepis vesicaria, Crepis xacintha, Euphorbia lagascae* and *Vernonia galamensis.* Additional species are not excluded.

In a particularly preferred embodiment of the present invention, the source organism is a
10 *Crepis sp.* comprising high levels of vernolic acid such as *Crepis palaestina,* amongst others or alternatively, *Vernonia galamensis.*

Wherein the isolated nucleic acid of the invention encodes a Δ6-epoxygenase or Δ9-epoxygenase enzyme or Δ12-epoxygenase or Δ15-epoxygenase enzyme, or at least
15 encodes an enzyme which is not involved in the direct epoxidation of arachidonic acid, the subject nucleic acid may be derived from any source producing said enzyme, including, but not limited to, yeasts, moulds, bacteria, insects, birds, mammals and plants.

The nucleic acid of the invention according to any of the foregoing embodiments may be
20 DNA, such as a gene, cDNA molecule, RNA molecule or a synthetic oligonucleotide molecule, whether single-stranded or double-stranded and irrespective of any secondary structure characteristics unless specifically stated.

Reference herein to a "gene" is to be taken in its broadest context and includes:
25 (i) a classical genomic gene consisting of transcriptional and/or translational regulatory sequences and/or a coding region and/or non-translated sequences (i.e. introns, 5'- and 3'- untranslated sequences);or
(ii) mRNA or cDNA corresponding to the coding regions (i.e. exons) and 5'- and 3'- untranslated sequences of the gene.

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The term "gene" is also used to describe synthetic or fusion molecules encoding all or part of a functional product. Preferred epoxygenase genes of the present invention may be derived from a natural epoxygenase gene by standard recombinant techniques. Generally, an epoxygenase gene may be subjected to mutagenesis to produce single or multiple
35 nucleotide substitutions, deletions and/or additions.

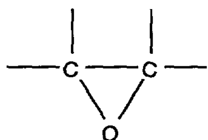
Insertions are those variants in which one or more nucleotides are introduced into a predetermined site in the nucleotide sequence, although random insertion is also possible with suitable screening of the resulting product. Nucleotide insertions include 5' and 3' terminal fusions as well as intra-sequence insertions of single or multiple nucleotides.

Deletions are variants characterized by the removal of one or more nucleotides from the sequence.

Substitutions are those variants in which at least one nucleotide in the sequence has been removed and a different nucleotide inserted in its place. Such a substitution may be "silent" in that the substitution does not change the amino acid defined by the codon. Alternatively, a conservative substitution may alter one amino acid for another similar acting amino acid, or an amino acid of like charge, polarity, or hydrophobicity.

In the context of the present invention, the term "fatty acid epoxygenase" shall be taken to refer to any enzyme or functional equivalent or enzymatically-active derivative thereof that catalyzes the biosynthesis of an epoxy fatty acid, by converting a carbon bond of a fatty acid to an epoxy group and preferably, by converting a carbon double bond of an unsaturated fatty acid to an epoxy group. Although not limiting the invention, a fatty acid epoxygenase may catalyze the biosynthesis of an epoxy fatty acid selected from the group consisting of: (i) 12,13-epoxy-9-octadecenoic acid (vernolic acid); (ii) 12,13-epoxy-9,15-octadecadienoic acid; (iii) 15,16-epoxy-9,12-octadecadienoic acid; (iv) 9,10-epoxy-12-octadecenoic acid; and (v) 9,10-epoxy-octadecanoic acid.

The term "epoxy", or "epoxy group" or "epoxy residue" will be known by those skilled in the art to refer to a three member ring comprising two carbon atoms and an oxygen atom linked by single bonds as follows:



Accordingly, the term "epoxide" refers to a compound that comprise at least one epoxy group as herein before defined.

Those skilled in the art are aware that fatty acid nomenclature is based upon the length of the carbon chain and the position of unsaturated carbon atoms within that carbon chain. Thus, fatty acids are designated using the shorthand notation:

5 $(\text{Carbon})_{\text{total}}(\text{carbon double bonds})_{\text{total}}^{\text{carbon double bond}(\Delta)\text{ position}}$,

wherein the double bonds are *cis* unless otherwise indicated. For example, palmitic acid (*n*-hexadecanoic acid) is a saturated 16-carbon fatty acid (i.e. 16:0), oleic acid (octadecenoic acid) is an unsaturated 18-carbon fatty acid with one double bond between C-9 and C-10 (i.e. $18{:}1^{\Delta 9}$), and linoleic acid (octadecadienoic acid) is an unsaturated 18-
10 carbon fatty acid with two double bonds between C-9 and C-10 and between C-12 and C-13 (i.e. $18{:}2^{\Delta 9,12}$).

However, in the present context an epoxygenase enzyme may catalyze the conversion of any carbon bond to an epoxy group or alternatively, the conversion of any double in an
15 unsaturated fatty acid substrate to an epoxy group. In this regard, it is well-known by those skilled in the art that most mono-unsaturated fatty acids of higher organisms are 18-carbon unsaturated fatty acids (i.e. $18{:}1^{\Delta 9}$), while most polyunsaturated fatty acids derived from higher organisms are 18-carbon fatty acids with at least one of the double bonds therein located between C-9 and C-10. Additionally, bacteria also possess C16- mono-unsaturated
20 fatty acids. Moreover, the epoxygenase of the present invention may act on more than a single fatty acid substrate molecule and, as a consequence, the present invention is not to be limited by the nature of the substrate molecule upon which the subject epoxygenase enzyme acts.

25 Preferably, the substrate molecule for the epoxygenase of the present invention is an unsaturated fatty acid comprising at least one double bond.

Furthermore, epoxygenase enzymes may act upon any number of carbon atoms in any one substrate molecule. For example, they may be characterized as Δ6-epoxygenase, Δ9-
30 epoxygenase, Δ12-epoxygenase or Δ15-epoxygenase enzymes amongst others. Accordingly, the present invention is not limited by the position of the carbon atom in the substrate upon which an epoxygenase enzyme may act.

The term "Δ6-epoxygenase" as used herein shall be taken to refer to an epoxygenase
35 enzyme which catalyzes the conversion of the Δ6 carbon bond of a fatty acid substrate to a

Δ6 epoxy group and preferably, catalyzes the conversion of the Δ6 double bond of at least one unsaturated fatty acid to a Δ6 epoxy group.

The term "Δ9-epoxygenase" as used herein shall be taken to refer to an epoxygenase enzyme which catalyzes the conversion of the Δ9 carbon bond of a fatty acid substrate to a Δ9 epoxy group and preferably, catalyzes the conversion of the Δ9 double bond of at least one unsaturated fatty acid to a Δ9 epoxy group.

As used herein, the term "Δ12-epoxygenase" shall be taken to refer to an epoxygenase enzyme which catalyzes the conversion of the Δ12 carbon bond of a fatty acid substrate to a Δ12 epoxy group and preferably, catalyzes the conversion of the Δ12 double bond of at least one unsaturated fatty acid to a Δ12 epoxy group.

As used herein, the term "Δ15-epoxygenase" shall be taken to refer to an epoxygenase enzyme which catalyzes the conversion of the Δ15 carbon bond of a fatty acid substrate to a Δ15 epoxy group and preferably, catalyzes the conversion of the Δ15 double bond of at least one unsaturated fatty acid to a Δ15 epoxy group.

The present invention clearly extends to genetic sequences which encode all of the epoxygenase enzymes *listed supra,* amongst others.

In one preferred embodiment of the invention, the isolated nucleic acid encodes a fatty acid epoxygenase enzyme which converts at least one carbon bond in palmitoleic acid ($16:1^{\Delta 9}$), oleic acid ($18:1^{\Delta 9}$), linoleic acid ($18:2^{\Delta 9,12}$), linolenic acid ($18:3^{\Delta 9,12,15}$), or arachidonic acid ($20:4^{\Delta 5,8,11,14}$) to an epoxy bond. Preferably, the carbon bond is a carbon double bond.

More preferably, the isolated nucleic acid of the invention encodes a fatty acid epoxygenase enzyme that at least converts one or both double bonds in linoleic acid to an epoxy group. According to this embodiment, an epoxygenase which converts both the Δ9 and the Δ12 double bonds of linoleic acid to an epoxy group may catalyze such conversions independently of each other such that said epoxygenase is a Δ9-epoxygenase and/or a Δ12-epoxygenase enzyme as herein before defined.

In an alternative preferred embodiment, the fatty acid epoxygenase of the present invention is a Δ12-epoxygenase, a Δ15- epoxygenase or a Δ9-epoxygenase as herein before defined.

5 More preferably, the fatty acid epoxygenase of the invention is a Δ12- epoxygenase as herein before defined.

In a particularly preferred embodiment of the invention, there is provided an isolated nucleic acid which encodes linoleate Δ12-epoxygenase, the enzyme which at least converts 10 the Δ12 double bond of linoleic acid to a Δ12-epoxy group, thereby producing 12,13-epoxy-9-octadecenoic acid (vernolic acid).

Although not limiting the present invention, the preferred source of the Δ12-epoxygenase of the invention is a plant, in particular *Crepis palaestina* or a further *Crepis sp.* which is 15 distinct from *C. palaestina* but contains high levels of vernolic acid, or *Vernonia galamensis*.

According to this embodiment, a Δ12-epoxygenase may catalyze the conversion of palmitoleic acid to 9,10-epoxy-palmitic acid and/or the conversion of oleic acid to 9,10-epoxy-stearic acid and/or the conversion of linoleic acid to any one or more of 9,10-epoxy-20 12-octadecenoic acid or 12,13-epoxy-9-octadecenoic acid or 9,10,12,13-diepoxy-stearic acid and/or the conversion of linolenic acid to any one or more of 9,10-epoxy-12,15-octadecadienoic acid or 12,13-epoxy-9,15-octadecadienoic acid or 15,16-epoxy-octadecadienoic acid or 9,10,12,13-diepoxy-15-octadecenoic acid or 9,10,15,16-diepoxy-12-octadecenoic acid or 12,13,15,16-diepoxy-9-octadecenoic acid or 9,10,12,13,15,16-25 triepoxy-stearic acid and/or the conversion of arachidonic acid to any one or more of 5,6-epoxy-8,11,14-tetracosatrienoic acid or 8,9-epoxy-5,11,14-tetracosatrienoic acid or 11,12-epoxy-5,8,14-tetracosatrienoic acid or 14,15-epoxy-5,8,11-tetracosatrienoic acid or 5,6,8,9-diepoxy-11,14-tetracosadienoic acid or 5,6,11,12-diepoxy-8,14-tetracosadienoic acid or 5,6,14,15-diepoxy-8,11-tetracosadienoic acid or 8,9,11,12-diepoxy-5,14-tetracosadienoic 30 acid or 8,9,14,15-diepoxy-5,11-tetracosadienoic acid or 11,12,14,15-diepoxy-5,8-tetracosadienoic acid or 5,6,8,9,11,12-triepoxy-14-tetracosenoic acid or 5,6,8,9,14,15-triepoxy-11-tetracosenoic acid or 5,6,11,12,14,15-triepoxy-8-tetracosenoic acid or 8,9,11,12,14,15-triepoxy-5-tetracosenoic acid, amongst others.

Those skilled in the art may be aware that not all substrates listed *supra* may be derivable from a natural source, but notwithstanding this, may be produced by chemical synthetic means. The conversion of both natural and synthetic unsaturated fatty acids to epoxy fatty acids is clearly within the scope of the present invention.

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The present invention is particularly directed to those epoxygenase enzymes that are mixed-function monooxygenase enzymes, and nucleic acids encoding said enzymes, and uses of said enzymes and nucleic acids. Accordingly, it is particularly preferred that the nucleic acid of the invention encode a fatty acid epoxygenase which is a mixed-function
10 monooxygenase enzyme.

In the context of the present invention, the term "mixed-function monooxygenase enzyme" shall be taken to refer to any epoxygenase polypeptide that comprises an amino acid sequence comprising three histidine-rich regions as follows:
15 (i) His-$(Xaa)_{3\text{-}4}$-His (SEQ ID NO: 21 and SEQ ID NO: 22);
(ii) His-$(Xaa)_{2\text{-}3}$-His-His (SEQ ID NO: 23 and SEQ ID NO: 24); and
(iii) His-$(Xaa)_{2\text{-}3}$-His-His (SEQ ID NO: 23 and SEQ ID NO: 24),

wherein His designates histidine, Xaa designates any naturally-occurring amino acid residue as set forth in Table 1 herein, the integer $(Xaa)_{3\text{-}4}$ refers to a sequence of amino
20 acids comprising three or four repeats of Xaa, and the integer $(Xaa)_{2\text{-}3}$ refers to a sequence of amino acids comprising two or three repeats of Xaa.

In the exemplification of the invention described herein, the inventors provide isolated cDNAs that comprise nucleotide sequences encoding the Δ12-epoxygenase polypeptides
25 of *Crepis palaestina* and *Vernonia galamensis*. Each exemplified full-length amino acid sequence encoded by said cDNAs which includes the three characteristic amino acid sequence motifs of a mixed-function monooxygenase enzyme as herein before defined. Close sequence identity between the amino acid sequences of the Δ12-epoxygenase enzymes from *C. palaestina* (SEQ ID NO: 2), an unidentified *Crepis sp* (SEQ ID NO: 4),
30 and *Vernonia galamensis* (SEQ ID NO: 20), suggests functional similarity between these polypeptides. In contrast, the amino acid sequences of these epoxygenases have lower identity to the amino acid sequences of a fatty acid desaturase or a fatty acid hydroxylase.

It is even more preferred that the epoxygenase of the present invention at least comprises a
35 sequence of amino acids which comprises three histidine-rich regions as follows:

(i) His-Glu-Cys-Gly-His-His (SEQ ID NO: 15);
(ii) His-Arg-Asn-His-His (SEQ ID NO: 16); and
(iii) His-Val-Met-His-His (SEQ ID NO: 17) or His-Val-Leu-His-His (SEQ ID NO: 18),

5 wherein His designates histidine, Glu designates glutamate, Cys designates cysteine, Gly designates glycine, Arg designates arginine, Asn designates asparagine, Val designates valine, Met designates methionine and Leu designates leucine.

The present invention clearly extends to epoxygenase genes derived from other species, 10 including the epoxygenase genes derived from *Chrysanthemum spp.* and *Euphorbia lagascae*, amongst others.

In a preferred embodiment, whilst not limiting the present invention, the epoxygenase genes of other species which are encompassed by the present invention encode mixed-15 function monooxygenase enzymes. The present invention further extends to the isolated or recombinant polypeptides encoded by such genes and uses of said genes and polypeptides.

The invention described according to this embodiment does not encompass nucleic acids 20 which encode enzyme activities other than epoxygenase activities as defined herein, in particular the $\Delta 12$-desaturase enzymes derived from *Arabidopsis thaliana, Brassica juncea, Brassica napus* or *Glycine max,* amongst others, which are known to contain similar histidine-rich motifs.

25 In the present context, "homologues" of an amino acid sequence refer to those amino acid sequences or peptide sequences which are derived from polypeptides, enzymes or proteins of the present invention or alternatively, correspond substantially to the amino acid sequences listed *supra*, notwithstanding any naturally-occurring amino acid substitutions, additions or deletions thereto.

30

For example, amino acids may be replaced by other amino acids having similar properties, for example hydrophobicity, hydrophilicity, hydrophobic moment, antigenicity, propensity to form or break α-helical structures or β-sheet structures, and so on. Alternatively, or in addition, the amino acids of a homologous amino acid sequence may be replaced by other

amino acids having similar properties, for example hydrophobicity, hydrophilicity, hydrophobic moment, charge or antigenicity, and so on.

Naturally-occurring amino acid residues contemplated herein are described in Table 1.

5

A homologue of an amino acid sequence may be a synthetic peptide produced by any method known to those skilled in the art, such as by using Fmoc chemistry.

Alternatively, a homologue of an amino acid sequence may be derived from a natural
10 source, such as the same or another species as the polypeptides, enzymes or proteins of the present invention. Preferred sources of homologues of the amino acid sequences listed *supra* include any of the sources contemplated herein.

"Analogues" of an amino acid sequence encompass those amino acid sequences which are
15 substantially identical to the amino acid sequences listed *supra* notwithstanding the occurrence of any non-naturally occurring amino acid analogues therein.

Preferred non-naturally occurring amino acids contemplated herein are listed below in Table 2.

20

The term "derivative" in relation to an amino acid sequence shall be taken to refer hereinafter to mutants, parts, fragments or polypeptide fusions of the amino acid sequences listed *supra*. Derivatives include modified amino acid sequences or peptides in which ligands are attached to one or more of the amino acid residues contained therein, such as
25 carbohydrates, enzymes, proteins, polypeptides or reporter molecules such as radionuclides or fluorescent compounds. Glycosylated, fluorescent, acylated or alkylated forms of the subject peptides are also contemplated by the present invention. Additionally, derivatives may comprise fragments or parts of an amino acid sequence disclosed herein and are within the scope of the invention, as are homopolymers or heteropolymers
30 comprising two or more copies of the subject sequences.

Procedures for derivatizing peptides are well-known in the art.

Substitutions encompass amino acid alterations in which an amino acid is replaced with a
35 different naturally-occurring or a non-conventional amino acid residue. Such substitutions

may be classified as "conservative", in which case an amino acid residue is replaced with another naturally-occurring amino acid of similar character, for example Gly⇔Ala, Val⇔Ile⇔Leu, Asp⇔Glu, Lys⇔Arg, Asn⇔Gln or Phe⇔Trp⇔Tyr.

5 Substitutions encompassed by the present invention may also be "non-conservative", in which an amino acid residue which is present in a repressor polypeptide is substituted with an amino acid having different properties, such as a naturally-occurring amino acid from a different group (e.g. substituted a charged or hydrophobic amino acid with alanine), or alternatively, in which a naturally-occurring amino acid is substituted with a non-
10 conventional amino acid.

Amino acid substitutions are typically of single residues, but may be of multiple residues, either clustered or dispersed.

15 Amino acid deletions will usually be of the order of about 1-10 amino acid residues, while insertions may be of any length. Deletions and insertions may be made to the N-terminus, the C-terminus or be internal deletions or insertions. Generally, insertions within the amino acid sequence will be smaller than amino-or carboxyl-terminal fusions and of the order of 1-4 amino acid residues.

20

The present invention clearly extends to the subject isolated nucleic acid when integrated into the genome of a cell as an addition to the endogenous cellular complement of epoxygenase genes. Alternatively, wherein the host cell does not normally encode enzymes required for epoxy fatty acid biosynthesis, the present invention extends to the
25 subject isolated nucleic acid when integrated into the genome of said cell as an addition to the endogenous cellular genome.

TABLE 1

Amino Acid	Three-letter Abbreviation	One-letter Symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Any amino acid as above	Xaa	X

TABLE 2

Non-conventional amino acid	Code	Non-conventional amino acid	Code
α-aminobutyric acid	Abu	L-N-methylalanine	Nmala
α-amino-α-methylbutyrate	Mgabu	L-N-methylarginine	Nmarg
aminocyclopropane-carboxylate	Cpro	L-N-methylasparagine	Nmasn
		L-N-methylaspartic acid	Nmasp
aminoisobutyric acid	Aib	L-N-methylcysteine	Nmcys
aminonorbornyl-carboxylate	Norb	L-N-methylglutamine	Nmgln
		L-N-methylglutamic acid	Nmglu
cyclohexylalanine	Chexa	L-N-methylhistidine	Nmhis
cyclopentylalanine	Cpen	L-N-methylisolleucine	Nmile
D-alanine	Dal	L-N-methylleucine	Nmleu
D-arginine	Darg	L-N-methyllysine	Nmlys
D-aspartic acid	Dasp	L-N-methylmethionine	Nmmet
D-cysteine	Dcys	L-N-methylnorleucine	Nmnle
D-glutamine	Dgln	L-N-methylnorvaline	Nmnva
D-glutamic acid	Dglu	L-N-methylornithine	Nmorn
D-histidine	Dhis	L-N-methylphenylalanine	Nmphe
D-isoleucine	Dile	L-N-methylproline	Nmpro
D-leucine	Dleu	L-N-methylserine	Nmser
D-lysine	Dlys	L-N-methylthreonine	Nmthr
D-methionine	Dmet	L-N-methyltryptophan	Nmtrp
D-ornithine	Dorn	L-N-methyltyrosine	Nmtyr
D-phenylalanine	Dphe	L-N-methylvaline	Nmval
D-proline	Dpro	L-N-methylethylglycine	Nmetg
D-serine	Dser	L-N-methyl-t-butylglycine	Nmtbug
D-threonine	Dthr	L-norleucine	Nle
D-tryptophan	Dtrp	L-norvaline	Nva
D-tyrosine	Dtyr	α-methyl-aminoisobutyrate	Maib
D-valine	Dval	α-methyl-γ-aminobutyrate	Mgabu
D-α-methylalanine	Dmala	α-methylcyclohexylalanine	Mchexa
D-α-methylarginine	Dmarg	α-methylcylcopentylalanine	Mcpen

	D-α-methylasparagine	Dmasn	α-methyl-α-napthylalanine	Manap
	D-α-methylaspartate	Dmasp	α-methylpenicillamine	Mpen
	D-α-methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
	D-α-methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
5	D-α-methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Norn
	D-α-methylisoleucine	Dmile	N-amino-α-methylbutyrate	Nmaabu
	D-α-methylleucine	Dmleu	α-napthylalanine	Anap
	D-α-methyllysine	Dmlys	N-benzylglycine	Nphe
	D-α-methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	Ngln
10	D-α-methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
	D-α-methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
	D-α-methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
	D-α-methylserine	Dmser	N-cyclobutylglycine	Ncbut
	D-α-methylthreonine	Dmthr	N-cycloheptylglycine	Nchep
15	D-α-methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
	D-α-methyltyrosine	Dmty	N-cyclodecylglycine	Ncdec
	D-α-methylvaline	Dmval	N-cylcododecylglycine	Ncdod
	D-N-methylalanine	Dnmala	N-cyclooctylglycine	Ncoct
	D-N-methylarginine	Dnmarg	N-cyclopropylglycine	Ncpro
20	D-N-methylasparagine	Dnmasn	N-cycloundecylglycine	Ncund
	D-N-methylaspartate	Dnmasp	N-(2,2-diphenylethyl) glycine	Nbhm
	D-N-methylcysteine	Dnmcys	N-(3,3-diphenylpropyl) glycine	Nbhe
25	D-N-methylglutamine	Dnmgln	N-(3-guanidinopropyl) glycine	Narg
	D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr
	D-N-methylhistidine	Dnmhis	N-(hydroxyethyl))glycine	Nser
30	D-N-methylisoleucine	Dnmile	N-(imidazolylethyl)) glycine	Nhis
	D-N-methylleucine	Dnmleu	N-(3-indolylyethyl) glycine	Nhtrp
	D-N-methyllysine	Dnmlys	N-methyl-γ-aminobutyrate	Nmgabu
	N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmet
35	D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpen

EXAMPLE 8

Fatty acid analysis of Cpal2 transgenic Linola plants

The binary plasmid construct described above comprising the Cpal2 cDNA clone (Figure 5) was transformed into *Agrobacterium tumefaciens* strain AGL1, using electroporation.
5 The transformed *A. tumefaciens* was used to infect *Linum usitatissimum* var. Eyre explants as described by Lawrence *et al* (1989), except that MS media was used as the basal medium for the induction of roots on regenerated shoot material.

Two primary Linola transformants (T_0 plants) designated AP20 and AP21 were confirmed
10 as being transgenic by PCR using primers directed against the Cpal2 gene and by showing that these plants were kanamycin resistant. Ten T1 seeds from each plant were analyzed individually for fatty acid composition using standard techniques.

As shown in Table 7, seed from AP20 segregated into 3 classes, comprised of three
15 seeds with no vernolic acid, two having greater than 0.7% vernolic acid, and five having intermediate levels (0.13-0.47%) of vernolic acid.

Similarly, seeds from AP21 segregated into 3 classes comprised of five seeds having no vernolic acid, two having greater than 0.25% vernolic acid and three having an
20 intermediate level (0.09-0.14%) of vernolic acid (Table 8).

Thus, a total of twelve seeds were obtained which contained vernolic acid. Eight of the twelve AP20 and AP21 seeds containing vernolic acid also contained 12,13-epoxy-9,15-octadecadienoic acid.

25

TABLE 7

Fatty acid composition of 10 individual T1 seeds from

Linola *Cpal2* primary transformant AP20

T_1 seed	Non-epoxy fatty acids									Epoxy fatty acids	
	16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1	18:1	18:2
1	6.4	3.6	17.8	68.1	2.0	0.2	-	0.6	-	-	-
2	6.0	3.5	25.4	60.8	1.4	0.2	0.2	-	-	0.70	0.23
3	6.0	3.9	20.4	64.6	2.1	0.3	0.6	-	-	-	-
4	6.3	3.5	28.3	57.3	1.3	0.2	0.2	1.4	-	0.34	0.28
5	5.2	4.8	24.9	61.2	1.6	0.3	0.2	0.1	-	0.37	-
6	5.8	4.1	23.3	63.1	1.9	0.2	0.2	0.2	-	0.47	-
7	5.9	4.3	21.7	64.1	2.2	0.2	0.2	0.2	-	0.13	0.12
8	5.9	3.3	22.3	65.2	2.0	0.2	0.2	0.1	0.2	-	-
9	5.6	4.0	25.2	61.4	1.7	0.2	0.2	0.1	-	0.84	-
10	6.2	4.4	27.4	57.9	1.7	0.2	0.2	0.2	-	0.54	-

TABLE 8

Fatty acid composition of 10 individual T1 seeds from

Linola *Cpal2* primary transformant AP21

T_1 seed	Non-epoxy fatty acids									Epoxy fatty acids	
	16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1	18:1	18:2
1	6.1	4.2	35.2	50.8	1.3	-	-	-	2.0	-	-
2	5.7	5.0	32.9	53.3	1.4	0.2	0.2	0.2		0.14	0.21
3	5.9	4.0	35.1	50.8	1.3	0.2	0.2	0.1	1.5	-	-
4	7.5	4.1	38.8	45.5	1.2	0.2	0.3	-	1.7	-	-
5	5.8	5.0	28.8	57.3	1.3	0.2	0.2	0.1	-	0.37	0.06
6	5.8	5.0	44.1	41.4	1.4	0.2	0.2	0.2	-	-	-
7	6.5	4.5	27.9	58.6	1.3	0.2	0.1	0.1	-	-	-
8	6.9	4.6	37.6	48.1	1.2	-	-	-	-	0.10	0.19
9	6.2	4.7	33.7	52.1	1.3	0.2	0.2	0.2	-	0.09	0.07
10	6.1	4.8	29.7	56.6	1.3	0.2	0.2	0.1	-	0.25	0.04

Four T1 plants were established from the kanamycin-resistant seedlings of AP20. All four plants were subsequently shown to produce vernolic acid in their T2 seed (Table 9). Levels of 18:2 epoxy fatty acids were not analyzed in these T2 seed.

TABLE 9

Fatty acid composition of T2 seeds from Linola *Cpal2* T1 progeny of AP20

T_2 seed	Non-epoxy fatty acids									epoxy fatty acid
	16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1	18:1
A	3.4	3.0	27.4	65.5	0.6	na	na	na	na	0.06
B	3.5	3.1	30.2	62.6	0.6	na	na	na	na	0.07
C	3.6	2.7	33.3	59.8	0.6	na	na	na	na	0.07
D	3.4	3.1	28.2	64.6	0.6	na	na	na	na	0.11

na. = not analyzed

5

EXAMPLE 9

Producing epoxy fatty acids in transgenic organisms

10 Production of an oil rich in vernolic acid was achieved by transforming the epoxygenase gene described herein, in particular SEQ ID NO: 1, into *Arabidopsis thaliana*, as described in the preceding Examples. As shown in Table 5, transgenic *A. thaliana* lines expressing SEQ ID NO: 1 produce high levels of vernolic acid in their seeds relative to other fatty acids. In particular, in one transgenic line (Cpal-17), the vernolic acid produced is as much 15 as 15.2% (w/w) of total seed fatty acid content.

Production of an oil rich in vernolic acid is also achieved by transforming the epoxygenase gene described herein, in any one of SEQ ID NOs: 1, 3, 5, or 19 and preferably any one of SEQ ID NOs:1 or 3 or 5 or 19, into any oil accumulating organism that normally has very 20 high levels of linoleic acid and minimal other competing enzyme activities capable of utilizing linoleic acid as a substrate. The genetic sequences of the invention are placed operably under the control of a promoter which produces high-level expression in oilseed, for example the napin seed-specific promoter.

In one alternative approach to the transformation of *A. thaliana*, high-linoleic genotypes of flax, sunflower, corn or safflower are transformed with the epoxygenase of the invention. High levels of vernolic acid are produced by the transgenic plants during seed oil synthesis, when the epoxygenase gene is expressed at high levels.

5

Alternatively, Linola™ (= low linolenic acid) flax is transformed with the epoxygenase of the invention. High levels of vernolic acid are produced by the transgenic Linola™ flax plants during seed oil synthesis, when the epoxygenase gene is expressed at high levels.

10 Additionally, the inventors have shown that labeled vernolic acid fed to developing flax seeds is not degraded but is incorporated into storage lipids at all three positions of the triglyceride molecule (see Example 10). Consistent with these data, high levels of vernolic acid synthesized by the introduced epoxygenase are readily deposited into the seed oil triglycerides of this species.

15

EXAMPLE 10

Incorporation of oleic acid and vernolic acid into the lipids of developing linseed cotyledons

20

Detached developing linseed cotyledons (six pairs in each incubation, duplicate incubations) at mid stage of seed development (20 days after flowering) were incubated with 10 nmol of the ammonium salts of either [1-^{14}C]vernolic acid (specific activity 3000 d.p.m./nmol) or [1-^{14}C]oleic acid (specific activity 5000 d.p.m./nmol) in 0.2 ml phosphate

25 buffer pH 7.2 for 30 min at 30□C. The cotyledons were then rinsed three times with 1 ml of distilled water and either extracted immediately in an Ultra Turrax according to Bligh and Dyer (1959) or incubated further in 0.5 m. 0.1 M phosphate buffer pH 7.2 for 90 or 270 min before extraction. An aliquot of the lipids in the chloroform phase was methylated and separated on silica gel TLC plates in n-hexane/diethylether/acetic acid (85:15:1). The

30 rest of the lipids in the chloroform phase of each sample were applied on two separate silica gel TLC plates and the plates were developed in chloroform/methanol/acetic acid/water (85:15:10:3.5 by vol) for polar lipids separation and in n-hexane/diethylether/acetic acid (60:40:1.5) for neutral lipid separation. Lipid areas with

migration corresponding to authentic standards were removed and radioactivity in each lipid were quantified by liquid scintillation counting.

The recovery of ^{14}C-label in the chloroform phase is depicted in Figure 8. Somewhat more than half of added radioactivity from both [^{14}C]oleic acid and [^{14}C]vernolic acid was taken up by the cotyledons and recovered as lipophilic substances after the 30 min pulse labeling. This quantity remained virtually unchanged during the further 270 min of incubation with both substrates. Separation of radioactive methyl esters of the lipids showed that most of the radioactivity (92%) from [^{14}C]vernolic acid feeding experiments resided in compounds with the same migration as methyl-vernoleate indicating that the epoxy group remained intact in the linseed cotyledons throughout the 270 min incubation.

About 28% of the activity from [^{14}C]vernolic acid feeding which was present in the chloroform phase resided in phosphatidylcholine after 30 min and the radioactivity decreased to only 5% at 300 min of incubation (Figure 9).

About 22% of the activity from [^{14}C]oleic acid feeding which was present in the chloroform phase resided in phosphatidylcholine after 30 min and the radioactivity decreased to about 11% at 300 min of incubation (Figure 9).

About 32% of the activity from [^{14}C]vernolic acid feeding which was present in the chloroform phase resided in triacylglycerols after 30 min and the radioactivity increased to over 60% at 300 min of incubation (Figure 10). The diacylglycerols contained some 24% of the activity in the [14C]vernolic acid feeding experiments and this quantity remained rather constant over the incubation periods.

About 5% of the activity from [^{14}C]oleic acid feeding which was present in the chloroform phase resided in triacylglycerols after 30 min and the radioactivity increased to 18% at 300 min of incubation (Figure 10). The diacylglycerols contained some 19% of the activity after 30 min in the [^{14}C]oleic acid feeding experiments and this quantity remained rather constant over the incubation periods.

The above experiment shows that linseed cotyledons do not metabolize the epoxy group of vernolic acid to any great extent. Further it shows that linseed cotyledons possess

mechanisms to efficiently remove vernolic acid from membrane lipids and incorporate them into triacylglycerols.

5 EXAMPLE 11

Cloning of Δ12-epoxygenase genes from an unidentified *Crepis* species

Homologues of the Cpal2 Δ12-epoxygenase gene were obtained from species other than *C. palaestina* which are rich in epoxy fatty acids, by cloning the members of the gene family of Δ12 mixed function monooxygenases that are highly expressed in developing 10 seeds and comparing their amino acid sequence to those of known Δ12-desaturase and Δ12-epoxygenase sequences.

Such genes were cloned either by screening developing seed cDNA libraries with genetic probes based on either the Cpal2 gene (SEQ ID NO: 1) or the D12V fragment (SEQ ID 15 NO: 7), or by amplifying PCR fragments using primers designed against conserved sequences of the plant Δ12 mixed function monooxygenases, as described herein. Putative Δ12-epoxygenase sequences show greater overall sequence identity to the Δ12-epoxygenase sequences disclosed herein, than to the known Δ12-desaturase sequences.

20 In one example of this approach, a full-length Δ12-epoxygenase-like sequence was obtained from an unidentified *Crepis sp.* containing high levels of vernolic acid in its seed oils and known not to be *Crepis palaestina*. Poly(A)+ RNA was isolated from developing seeds of this *Crepis sp.* using a QuickPrep Micro mRNA purification kit (Pharmacia Biotechnology) and used to synthesize an oligosaccharide d(T)-primed double-stranded 25 cDNA. The double stranded cDNA thus obtained was then ligated to *Eco*R1/ *Not*I adaptors (Pharmacia Biotechnology) and a cDNA library was constructed using the ZAP-cDNA Gigapack cloning kit (Stratagene). The cDNA library on Hybond N+ membrane filters (Amersham) was screened with the random-labeled D12V fragment (SEQ ID NO: 7) derived from *Crepis alpina* as prescribed by the manufacturer, using standard 30 hybridization conditions. This resulted in the purification of a recombinant bacteriophage designated CrepX.

The nucleotide sequence of the CrepX cDNA was determined and is set forth in SEQ ID NO: 3. The deduced amino acid sequence of CrepX (SEQ ID NO: 4) comprises a 374

O9059769.100701

amino acid protein having 97% identity to the Cpal2 Δ12-epoxygenase sequence, but only 57% identity to the *Arabidopsis thaliana* L26296 Δ12-desaturase sequence. This clearly demonstrates the presence of a gene in another *Crepis sp.* having high vernolic acid content, which gene is highly homologous to the Cpal2 Δ12-epoxygenase gene and is clearly not a desaturase gene.

EXAMPLE 12

Cloning of Δ12-epoxygenase genes from *Vernonia galamensis*

Following the general strategy outlined in the preceding example, a homologue of the Cpal2 Δ12-epoxygenase gene was also obtained from *Vernonia galamensis*, containing high levels of vernolic acid in its seeds.

A partial Δ12-epoxygenase-like sequence was obtained from *V. galamensis*, by preparing first strand cDNA templates using total RNA from developing seeds as a template. A PCR fragment (550 nucleotides in length), designated as Vgal1, was obtained by amplifying the single-stranded cDNA, using primers derived from the deduced amino acid sequence of plant mixed function monooxygenases. The nucleotide sequence of the amplified DNA was determined using standard procedures and is set forth in SEQ ID NO: 5.

Alignment of the deduced amino acid sequence of the Vgal1 PCR fragment (SEQ ID NO: 6) with the full sequence of Cpal2 Δ12-epoxygenase and the *Arabidopsis thaliana* L26296 Δ12-desaturase (Figure 2) demonstrates that the amplified Vgal1 sequence encodes an amino acid sequence corresponding to the region spanning amino acid residues 103-285 of the Cpal2 polypeptide. Within this region, the Vgal1 sequence showed greater amino acid identity with the Cpal2 Δ12-epoxygenase sequence (67%) than with the *A. thaliana* Δ12-desaturase sequence (60%), suggesting that the amplified DNA corresponds to an epoxygenase rather than a desaturase sequence.

The corresponding full-length Δ12-epoxygenase sequence was obtained from *V. galamensis,* and the nucleotide sequence of the full-length clone determined (SEQ ID NO: 19). The deduced amino acid sequence of the full-length Vgal Δ12-epoxygenase polypeptide (SEQ ID NO: 20) comprises 384 amino acids comprising all three conserved

mixed function monooxygenase consensus sequences for epoxygenases as set forth in SEQ ID NOs: 15, 16, and 18 (see Figure 2).

5

EXAMPLE 13

Demonstration of epoxygenase activity for the *V. galamensis* clone

Confirmation that the full-length cDNA clone of *V. galamensis* encodes an epoxygenase is obtained by transforming *Arabidopsis thaliana* with a binary vector comprising the isolated cDNA clone in the sense orientation and in operable connection with a promoter as
10 described in the preceding examples. Transformed tissue is examined for the presence of epoxy fatty acids that *A. thaliana* would not otherwise produce. Additionally, the level of hydroxy fatty acids is determined, as such fatty acids can be formed from the metabolism of an epoxy fatty acid, by the action of endogenous *A. thaliana* epoxide hydrolases (Blee and Schuber, 1990).

15

The *V. galamensis* cDNA (SEQ ID NO: 19) was cloned into a binary vector construct, such as that shown in Figure 4, essentially as described in the preceding examples. The Binary plasmid harboring SEQ ID NO: 19 was transformed into *Agrobacterium* strain AGLI using electroporation and used to transform *A. thaliana*. Transgenic *A. thaliana* plants
20 were obtained according to the method described by Valvekens *et al.* (1988) and Dolferus *et al.* (1994).

Transgenic plants and untransformed (i.e. control) plants are grown to maturity. Mature seed of each plant are analyzed for fatty acid composition by standard techniques.
25 Primary transformant (T_0) plants are established and T1 seed are harvested from each plant and analyzed for their fatty acid composition by gas chromatography. T_0 plants are shown to contain higher levels of epoxy fatty acids in their T1 seed lipids than the seeds of untransformed control plants.

30 Gas chromatography of methyl esters prepared from seed lipids of T1 seed of *Vgal* transformed *Arabidopsis thaliana* plants is performed to show the presence of additional fatty acids compared to the untransformed controls. The retention time of these compounds permits their identification as epoxy fatty acids, and/or derivatives of epoxy

fatty acids that are produced by the action of endogenous desaturase enzymes on the epoxy fatty acids.

Confirmation of the exact identity of the epoxy fatty acid products and derivatives thereof
5 is obtained by mass spectroscopy of the diols from the epoxy fatty acid fraction of transformed plants. The diols are converted further to trimethylsilyl ethers and analyzed by GC-MS DB23 on a fused silica capillary column (Hewlett-Packard 5890 II GC coupled to a Hewlett Packard 5989A MS working in electron impact at 70eV15).

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